PATENT SPECIFICATION (11) 1404306

1404306

(21) Application No. 7933/74 (22) Filed 21 Feb. 1974
(31) Convention Application No. 2602/73 (32) Filed 22 Feb. 1973 in
(33) Switzerland (CH)
(44) Complete Specification published 28 Aug. 1975
(51) INT CL* C07G 7/02 C07D 309/12
(52) Index at acceptance
C3H H1 Z
C2C 1672 215 220 22Y 253 25Y 30Y 351 355 360 361 362 364 365 36Y 388 509 50Y 625 634 643 662 665 672 699 UL

(19)

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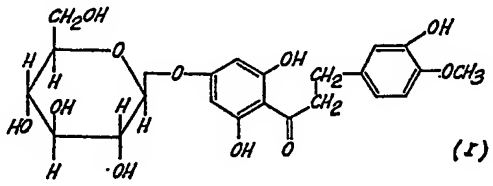
(54) MANUFACTURE OF HESPERETIN DIHYDROCHALCONE GLUSOCIDE

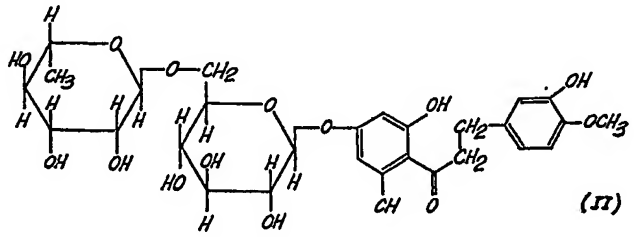
(71) We, L. GIVAUDIN & CIE, Societe Anonyme, a Swiss Company of Verner-Geneve, Switzerland, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement: —

The present invention is concerned with a process for the manufacture of hesperetin dihydrochalcone glucoside.

It is known that the flavourless glycoside hesperidin, which is principally present in the peel of citrus fruits, can be converted into the very sweet (and consequently usable as a sweetener) hesperetin dihydrochalcone glucoside of the formula



by treatment with alkali, hydrogenation and enzymatic hydrolysis (see, for example, United States Patent Specification No. 3,583,894). In so doing, the hesperidin dihydrochalcone of the formula



is subjected to the action of the enzyme system of naringinase, the β-glucosidase activity of which has been extensively destroyed so that it predominantly cleaves only the rhamnose. The inactivation of the β-glucosidase of the enzyme system is carried out by heating an aqueous naringinase suspension to a temperature of about 60—65°C for 30—120 minutes at a pH of 6.4—6.8. This procedure is, however, associated with a partial loss of rhamnosidase activity, the absolute magnitude of which is not reproducible.

It has now surprisingly been found in accordance with the present invention that by bonding a glycolytic enzyme or enzyme system having rhamnosidase activity, such as is exhibited, for example, by naringase and hesperidinase, to a solid carrier, especially to a solid activated carrier, the β-glucosidase activity is selectively blocked.

The present invention is based on the foregoing finding and is accordingly concerned with a process for the manufacture of hesperetin dihydrochalcone glucoside from hesperidin dihydrochalcone by means of glycolytic enzymes or enzyme systems having rhamnosidase activity, such as is exhibited by hesperidinase and naringinase, said process being carried out by contacting hesperidin dihydrochalcone with a solid carrier-bonded enzyme or enzyme system having inhibited β-glucosidase activity. The invention is also concerned with a glycolytic enzyme or enzyme system derivative having rhamnosidase activity and inhibited β-glucosidase activity, which has extensively lost the β-glucosidase activity, especially with respect to the hesperidin dihydrochalcone as the substrate, by coupling of the enzyme or enzyme system to a solid carrier material, as well as its manufacture.

An enzyme or enzyme system which is preferred in the present invention is hesperidinase, a commercially available product.

Suitable solid carrier materials which are used for the manufacture of enzyme preparations in accordance with the present invention, especially hesperidinase having inhibited β-glucosidase activity, are polymers having an organic or inorganic basis. Examples of materials having an inorganic basis are natural and synthetic silicates and silica-containing substances such as, for example, glass, sand, silica gel, kieselguhr, bentonite and wollastonite as well as metal oxides such as aluminium oxide and hydroxylapatite. Suitable polymers having an organic basis are, for example, cellulose and polyamides. In an especially preferred embodiment of the present invention, there can be used as the carrier material porous glass; for example, in the form in which it is commercially available, preferably with a particle size of about 80—400 mesh (U.S. Standard Sieve) and an average pore diameter of about 75—2000 Å. The carrier material can, however, also have another form and/or size if the conditions should require this.

The solid carrier material is expediently covered with a layer of an aromatic diamine. This can advantageously be carried out by treatment with a solution of the amine, if desired with slight warming. Suitable aromatic diamines are, for example, 1,3 - phenylenediamine, 4,4′ - diamino - diphenyl, 4,4′ - diamino - stilbene and 4,4′ - diamino - diphenyl - methane as well as the correspondingly substituted compounds. 4,4′ - diamino - diphenyl - methane is the especially preferred diamine. The thus-obtained product is then diazotised in a manner known *per se;* for example, by reaction with a nitrite and an acid (e.g. hydrochloric acid or acetic acid), expediently at a temperature between 0°C and room temperature. In accordance with the previously described procedure, the carrier particles are given a strongly adhering covering which is activated for the bonding of the protein.

In order to bond the enzyme to the activated solid carrier, the carrier is treated with a solution of the enzyme, a ratio of protein/carrier of 1—50 mg/g being expediently maintained. There may be used as the solvent for the enzyme any solvent which is customarily used in enzyme chemistry, but buffer solutions (e.g. phosphate or citrate buffers) are preferably used. The reaction of the enzyme with the diazotised carrier material is preferably carried out in a buffer solution having a pH of between 4.5 and 8.0 while shaking for about 30 minutes to 24 hours, preferably for 1—2 hours, and at a temperature between 0°C and 30°C. When hesperidinase is used, a buffer having a pH of 6.0 to 7.5 is especially preferred and the preferred reaction temperature is ca 4°C.

The thus-obtained enzyme which is bonded to a solid activated carrier and the β-glucosidase activity of which is selectively inhibited, is suitable for the degradation of hesperidin dihydrochalcone to hesperetin dihydrochalcone glucoside. In addition, the thus-obtained insolubilised enzyme has known advantageous properties; it can be readily separated from the substrate (e.g. by filtration or by centrifuging), it can be used several times and it is especially suitable for use in continuous processing. Finally, the enzyme preparation obtained according to the present invention has a high stability. In the case of hesperidinase bonded to porous glass, there is observed an especially good activity and stability in a continuous process in columns such as is known, for example, in chromatography.

The reaction of the hesperidin dihydrochalcone with the carrier-bonded enzyme can be carried out in a manner known *per se;* namely in stationary plant (e.g. in stirred reactors) and, as mentioned earlier, advantageously in continuous plant (e.g. in reaction columns). In so doing, an initial substrate concentration of about 5% is preferably used, but solutions having higher or lower concentrations can, of course, also be used. The working-up of the reaction product does not pose any problems and it can be carried out in a known manner. For example, it can be carried out simply by concentrating the reaction mixture to dryness without further purification

since the aglycone which may be present in slight amounts as a by-product is also sweet and physiologically harmless.

The following Examples illustrate the present invention:

Example 1

Manufacture of the activated carrier

1 g of porous glass (120—200 mesh, U.S. Standard Sieve, pore diameter: 2000 Å) was added to a solution of 200 mg of 4,4' - diamino - diphenyl - methane in 10 ml of dry benzene. The mixture was warmed for 5 minutes in a water-bath (80°C) with occasional shaking, then evaporated under a vacuum until all the benzene had been removed, The residue was added to 20 ml of ice-cold water, treated while stirring with 300 mg of sodium nitrite and then with dilute hydrochloric acid (20 vol-%) to a pH of 1—2. The resulting mixture was then stirred for 10 minutes at 0°C, the pH adjusted to 5 by the addition of 1-N sodium hydroxide and the supernatant decanted off. The residue was washed several times with ice-cold water, which had been brought to pH 4—5 by the addition of hydrochloric acid, until the supernatant was colourless. The thus-obtained particles were used immediately for the enzyme bonding.

Example 2

Bonding of the enzyme

1 g of the carrier material manufactured according to the procedure described in Example 1 was added to 25 ml of an ice-cold solution of 2 mg of hesperidinase (5.2 units/mg) in 0.05-M phosphate buffer (pH 6.5—7.8) and shaken gently at 4°C for 1 hour. The mixture was then filtered, the particles were washed three times with 50 ml of 1-M potassium chloride solution each time and once with 50 ml of 0.05-M citrate buffer (pH 6.5), suspended in 50 ml of 0.05-H citrate buffer (pH 6.5) and then stored at 4°C.

Example 3

Conversion of hesperidin dihydrochalcone into hesperetin dihydrochalcone glucoside

2 g of hesperidinase (4.3 units) bonded to porous glass, manufactured according to the procedure described in Example 2, were shaken for 2 hours at 45°C with a solution of 500 mg of hesperidin dihydrochalcone in 20 ml of 0.05-M citrate buffer (pH 4.5). Chromatographic analysis of the resulting solution showed that, after this time, the hesperidin dihydrochalcone had been quantitatively converted into hesperetin dihydrochalcone glucoside, whereby traces of the corresponding aglycone could be detected. The carrier-bonded enzyme was filtered off, washed with 0.05-M citrate buffer (pH 6.5) at 45°C and used immediately or stored at 4°C for future use. The filtrate was combined with the wash-solutions and concentrated to dryness under reduced pressure. The residue was extracted three times with 50 ml of dioxane. After removal of the solvent, the extract yielded 240 mg (70% yield) of the very sweet hesperetin dihydrochalcone glucoside.

A similarly good yield of glucoside was obtained by a triple direct extraction of the reaction solution with ethyl acetate without previous concentration.

By cooling the reaction solution with ice, a crystalline separation of the glucoside is achieved. This working-up is, in fact, not quantitative, but well-suited to large batches where extraction methods are less suitable.

Example 4

Conversion of hesperidin dihydrochalcone into hesperetin dihydrocalcone glucoside

3 g of hesperidinase (8.1 units) bonded to porous glass, manufactured according to the procedure described in Example 2, were treated in a glass column (1.0×10 cm) with a solution of 50 mg of hesperidin dihydrochalcone in 0.05-M citrate (pH 4.5) at 45°C, a flow rate of 11 ml/hour being maintained. Continuous chromatographic analysis showed the quantitative degradation of the hesperidin dihydrochalcone to hesperetin dihydrocalcone glucoside and rhamnose as well as a trace of agylcone.

Example 5

Conversion of hesperidin dihydrochalcone into hesperetin dihydrochalcone glucoside

118 g of hesperidin dihydrochalcone were quantitatively hydrolysed in a reaction column containing 5 g of carrier-bonded hesperidinase (20 mg of enzyme) in a continuous operation over a period of 2 weeks with an initial flow rate of 20 ml/hour

which returned to 10 ml/hour at the end of the operation, without the activity of the enzyme being exhausted.

WHAT WE CLAIM IS:—

1. A process for the manufacture of hesperetin dihydrochalcone glucoside comprising contacting hesperidin dihydrochalcone with a solid carrier-bonded enzyme or enzyme system having rhamnosidase activity and having inhibited β-glucosidase activity.

2. A process according to claim 1, wherein a carrier-bonded hesperidinase having inhibited β-glucosidase activity is used.

3. A process according to claim 2, wherein the carrier is an activated silica-containing material.

4. A process according to claim 2 and claim 3, wherein the carrier consists of porous glass having a layer of a diazotised aromatic diamine.

5. A process according to claim 4, wherein said aromatic diamine is 4,4′ - diamino - diphenyl - methane.

6. A process for the manufacture of hesperetin dihydrochalcone glucoside, substantially as hereinbefore described with reference to Examples 3, 4 and 5.

7. Hesperetin dihydrochalcone glucoside, when manufactured by the process claimed in any one of claims 1 to 6 inclusive.

8. A process for the manufacture of a carrier-bonded enzyme or enzyme system derivative having rhamnosidase activity and inhibited β-glucosidase activity, which process comprises treating a carrier with a solution of an enzyme or enzyme system having rhamnosidase activity.

9. A process according to claim 8, wherein hesperidinase is used as the enzyme.

10. A process according to claim 8 and claim 9, wherein an activated silica-containing material is used as the carrier.

11. A process according to any one of claims 8 to 10 inclusive, wherein there is used as the carrier porous glass which is covered with a layer of diazotised aromatic diamine.

12. A process according to claim 11, wherein 4,4′ - diamino - diphenyl - methane is used as the aromatic diamine.

13. A process according to any one of claims 9 to 12 inclusive, wherein the reaction of the hesperidinase with the carrier is carried out in a buffer solution having a pH of 4.5—8.0.

14. A process according to claim 13, wherein the pH of the buffer solution is 7.5.

15. A process according to claim 13 or 14, wherein the reaction is carried out at about 4°C.

16. An enzyme derivative or enzyme system derivative having rhamnosidase activity and inhibited β-glucosidase activity which comprises the enzyme or enzyme system bonded to a carrier.

17. A derivative according to claim 16 which consists of hesperidinase bonded to a carrier.

18. A hesperidinase derivative according to claim 17, wherein the carrier is an activated silica-containing material.

19. A hesperidinase derivative according to claims 16 and 17, wherein the carrier consists of porous glass which is covered with a layer of diazotised aromatic diamine.

20. A hesperidinase derivative according to claim 18, wherein said aromatic diamine is 4,4′ - diamino - diphenyl - methane.

21. A hesperidinase derivative according to any one of claims 15 to 19 inclusive, wherein the bonding of the enzyme to the carrier is effected via azo groups.

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Printed for Her Majesty's Stationery Office, by the Courier Press, Leamington Spa, 1975.
Published by The Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.